Decreased Migration of Langerhans Precursor-Like Cells in Response to Human Keratinocytes Expressing HPV-16 E6/E7 is Related to Reduced Macrophage Inflammatory Protein-3α Production

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Abstract. Infection with high-risk human papillomavirus (HPV) types, particularly type 16 and 18, contributes to 90% of cervical cancer cases. HPV infects cutaneous or mucosal epithelium, tissue that is monitored for microbial infection or damage by Langerhans cells. In lesions produced by HPV type 16, there is a reduction in immune cells, especially Langerhans cells. Langerhans precursor cells selectively express CCR6, the receptor for macrophage inflammatory protein 3α (MIP-3α), and function as potent immune responders to inflamed epithelium and initiators of the innate immune response. It has been reported that E6 and E7 of high-risk HPVs interfere with immune mediators in order to suppress the recruitment of immune cells and antiviral activities of infected cells. Here we show that HPV-16 E6 and E7 inhibit MIP-3α transcription resulting in suppression of the migration of immature Langerhans precursor cells. Interestingly, the E6 and E7 proteins from the low-risk HPV types also inhibited MIP-3α transcription. These results suggest that one mechanism by which HPV-infected cells suppress the immune response may be through the inhibition of a vital alert signal, thus, contributing to the persistence of HPV infection.

Introduction. Human papillomaviruses (HPVs) are small DNA viruses which exhibit tropism for cutaneous or mucosal epithelium. Commonly categorized as either low or high-risk HPV types based on their oncogenic potential, infections with the high-risk HPV types such as HPV-16 and 18 have been implicated in cervical/ anogenital cancer and oral squamous cell carcinomas (41). The mucosal lesions caused by HPVs often resolve over time, and a high percentage of young women exposed to HPV clear the infection within a short period following initial contact with the virus (61). Empirical evidence for the critical role the immune response plays in

controlling an HPV infection comes from the high prevalence and persistence of HPV infections in immunosuppressed individuals (63). In general, the mucosal immune response to an infection begins with the cells of the epidermis secreting alarm signals which act on immune cells as well as the infected cells. Chemokines, cytokines, adhesion molecules and proteases direct the migration of leukocytes, monocytes, lymphocytes, neutrophils, eosinophils, basophils, natural killer cells, dendritic cells, and endothelial cells (55), while interferons inhibit viral replication within the infected cells (64).

Evasion of the immune response would contribute to the survival and propagation of HPV-infected cells. Two genes in the high-risk HPVs, E6 and E7, are associated with the virus's oncogenic potential to transform infected cells. In addition to their well-documented effects on cell cycle and differentiation, E6 and E7 also negatively impact the immune response by inhibiting the production of immune mediators. Expression of E6 is inversely correlated with the expression of interleukin-18 (IL-18), a pro-inflammatory cytokine similar to IL-1 that induces interferon-γ (IFN-γ) production (10). E6 and, to a lesser extent, E7 down-regulate the expression of IL-8, a chemoattractant for T cells (28). E6 and E7 also suppress the expression of a chemokine, monocyte chemoattractant protein-1 (MCP-1), in female genital epithelial cells (35). Additionally, E6 and E7 contribute to the escape from the antiviral and antiproliferative properties of tumor necrosis factor-α (TNF-α) and IFN-α through growth stimulatory effects, loss of TNF-sensitivity, and interaction with components of the IFN signaling pathway (61).

Following microbial infection, chemokines secreted by the epidermis initiate the adaptive immune response by recruiting dendritic cells. Dendritic cells are antigen-presenting cells which play an important role in the capture of antigen and stimulation of T lymphocytes (5). Many subsets of dendritic cells exist, characterized by differences in surface marker expression and

developmental pathways. All dendritic cells differentiate from bone marrow progenitors and circulate in peripheral blood as immature precursors. Langerhans cells (LCs) are the dendritic cells that reside in the epidermis and immature LCs, efficient in the uptake of antigens, continually monitor the epidermal microenvironment for damage and infection. Following antigen processing, LCs mature through differential regulation of surface markers and migrate to T cell areas of the body, such as dermal lymphatics or peripheral lymphoid organs (5, 53). Among the chemokines and cytokines secreted by epidermal cells, macrophage inflammatory protein-3α (MIP-3α) is the most potent chemokine for LC precursors and dermal dendritic cells (12). Originally discovered to be expressed in lung and liver tissue (56), liver and activation-induced chemokine (LARC)/CCL20/MIP-3α is also produced in intestinal epithelium and epidermal keratinocytes (14, 26), but primarily in areas of inflamed epithelium (9, 46). MIP-3α expression can be increased by TNF-α, IL-1α, or IL-1β in oral squamous cell carcinomas, intestinal epithelial cells, and keratinocytes (1, 12, 26, 32, 36, 46, 67). Immature dendritic cells, memory T cells, and epidermal LC precursors migrate in response to MIP-3α due to their selective expression of CC chemokine receptor 6, CCR6 (19, 37, 40, 54).

Investigations of cervical biopsies revealed that the number of LCs was significantly reduced in intraepithelial neoplasia and low-grade/high-grade lesions when compared to normal tissue (2, 11, 70). Thus, areas of HPV persistence exhibiting decreases in LC density suggest decreased immune surveillance. Our studies show that human keratinocytes expressing HPV-16 E6 and E7 produce less MIP-3α than control cells. The result of this lack of secreted MIP-3α is a reduction in the migration of Langerhans precursor-like cells. Our results suggest that the down-regulation of MIP-3α is one mechanism by which HPV infections could evade the immune response and persist undetected in the epithelium.

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Materials and Methods

Keratinocyte culture and preparation of cell lines

Primary human foreskin keratinocytes (HFKs) were isolated from neonatal foreskins and cultured in EpiLife medium supplemented with human keratinocyte growth supplement (bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, human epidermal growth factor) and penicillin/streptomycin/amphotericin B (PSA) solution (Cascade Biologics). Cells were cultured in EpiLife medium supplemented without bovine pituitary extract and hydrocortisone (hereafter called minimal EpiLife) when culture supernatants were harvested for use in ELISAs and migration assays.

Generation of stable cell lines expressing HPV-16 E6/E7, HPV-6 E6/E7, and HPV-11 E6/E7 was accomplished using the pBabe retroviral system (43). Retrovirus encoding either vector alone or E6/E7 from HPV-16, HPV-6, and HPV-11 was produced by transfecting ΦNX packaging cells (American Type Culture Collection) with vesicular stomatitis virus envelope gene and pBabe retroviral constructs by calcium phosphate method (BD Biosciences), according to the manufacturer's protocol. 48 h after transfection, retrovirus was concentrated by centrifugation for 90 min at 15,000 rpm, 4°C, and virus pellets were resuspended in 2mL EpiLife overnight at 4°C. Resuspended virus was either used immediately to infect HFKs or frozen at -80°C for future use. Resuspended virus was used to infect HFKs in 8μg polybrene per mL of EpiLife for 5 h. Cells were allowed to recover for 24 h and then selected with 1.25μg/mL puromycin for 2-4 days. Stable cell lines were expanded for treatment with polyinosine-polycytidylic acid [Poly(I)·Poly(C)] (Amersham Biosciences).

Generation of E6 and E7mutations

Mutagenesis of E6/E7 was performed on HPV-16 E6/E7 in pGEM7Zf using the

QuikChange kit (Stratagene). The following primers were used to generate a stop codon at 16th

amino acid in E7 gene of HPV-16 for E6/E7stop (forward

GATTTGTAACCAGAGACAACTG; reverse CAGTTGTCTCTGGTTACAAATC) and a stop

codon at 15th amino acid in E6 gene of HPV-16 for E6stop/E7 (forward

CCACAGTAATGCACAGAGCTGC; reverse GCAGCTCTGTGCATTACTGTGG). HPV-16

E6(C66G/C136G) and HPV-16 E7.2, previously characterized as unable to bind CBP/p300 and

pCAF (28, 49), were generated in combination with E7stop and E6stop respectively. The

primers listed previously were used to mutate HPV-16 E6(C66G/C136G)/E7 in pGEM7ZF to

generate E6(C66G/C136G)/E7stop. Mutagenesis of E7 was performed on HPV-16 E6stop/E7 in

pGEM7Zf using the QuikChange kit (Stratagene). The following primers were used to generate

an amino acid substitution of histidine to proline at position 2 (forward

GCTGTAATCATGCCTGGAGATACACCTACA; reverse

TGTAGGTGTATCTCCAGGCATGATTACAGC). The mutants were sequenced and subcloned

into the pBabe retroviral vector.

Measurement of MIP-3α by qPCR and ELISA

1 x 10^{6} cells were seeded on 10-cm dishes 24 h prior to treatment with Poly(I)·Poly(C).

Cells were treated with 100μg/mL Poly(I)·Poly(C) in antibiotic-free EpiLife for 16 h, washed 5

times with PBS, and refed with minimal EpiLife for 4 h. Cells were harvested for RNA

isolation, and culture supernatants were collected for use in ELISAs and migration assays.

Total RNA was extracted using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. cDNA amplication was carried out in 25µL RT reaction mixtures containing 3µg RNA, 400µM deoxyribonucleoside triphosphates, RNase out ribonuclease inhibitor (Invitrogen), 10ng random hexamer primer, 5U avian myeloblastosis virus RT (Promega), and 5X RT buffer (Promega). cDNA obtained was used in real-time PCR reaction mixtures containing 2.5µL each of (1µM) forward and reverse primers, 12.5µL 2X iQ SYBR Green Supermix (BioRad), and 1µL cDNA template. The protocol used included a denaturation step (95°C for 30 s) followed by amplification repeated 45 times (95°C for 30 s, 60°C for 1 min, 68°C for 1 min). A melt curve analysis performed following every run confirmed the amplification of a single product and no primer dimer. In each experiment, reactions were carried out in quadruplicate for each sample and glyceraldehyde phosphate dehydrogenase (GAPDH) was used for normalization. Primers used for real-time PCR were obtained from Primer Bank (71) [MIP-3α forward: TGCTGTACCAAGAGTTTGCTC, MIP-3α reverse: CGCACACAGACAACTTTTTCTTT, GAPDH forward: TGTTGCCATCAATGACCCCTT, GAPDH reverse: CTCCACGACGTACTCAGCG]. Paired t-test was used to generate p values.

MIP-3α was measured in culture supernatants collected from Poly(I)·Poly(C)-treated cells using the human MIP-3α DuoSet ELISA Development kit (R & D Systems) according to the manufacturer's instructions. The lower limit of detection was 32pg/mL. Each sample was run in triplicate in each experiment and paired t-test was used to generate p values.

Culture of Langerhans Precursor-like Cells (LPLCs)

Mononuclear cells were isolated from peripheral blood of healthy donors by centrifugation on Lymphocyte Separation Medium (Cellgro). The mononuclear cell layer was

collected from the interface and monocytes were purified by positive magnetic selection using CD14 microbeads and an MS separation column (Miltenyi Biotec). The purity of CD14^{+} cells was routinely >95%, as assessed by flow cytometry.

Purified CD14^{+} monocytes were cultured in 24-well tissue culture plates (Costar) in complete medium [RPMI 1640-supplemented with L-glutamine (Cellgro), 1% penicillin/streptomycin, and 10% heat-inactivated fetal calf serum (Fetalclone I, Hyclone)] supplemented with 200ng/mL GM-CSF and 10ng/mL TGF-β1 (Peprotech). At days 2 and 4, cells were fed with fresh medium plus cytokines. At day 6, LPLCs were harvested, analyzed by flow cytometry, and used in migration assays.

Analysis of LPLC surface marker expression and phagocytic capacity

1 x 10^5 cells were incubated for 30 min at 4°C in PBS, 2% fetal calf serum (Fetalclone I, Hyclone), 1% sodium azide, with allophycocyanin-conjugated CD14 (eBioscience), phycoerythrin-conjugated CD83 (eBioscience), phycoerythrin-conjugated CCR6 (BD PharMingen), or fluorescein isothiocyanate-conjugated HLA-DR (BD PharMingen) monoclonal antibodies at the appropriate concentration or with control isotype-matched irrelevant monoclonal antibodies at the same concentration. Cells were washed twice and analyzed with a FACS caliber (Becton-Dickinson) using CellQuest software.

1 x 10^5 cells were incubated with 10μg/mL fluorescein isothiocyanate-conjugated ovalbulmin (Molecular Probes) for 30 min at 37°C or 4°C (control cells). Cells were washed twice and analyzed with a FACS caliber. Fluorescein intensity provided a measurement of phagocytic capacity of the cells.

Migration assay

Migration assays were carried out using 24-well Transwell plates with 5-μm pore size polycarbonate filters (Costar 3421). Briefly, 2×10^5 LPLCs in 100μL medium were added to the upper wells and 600μL of conditioned medium was added to the lower wells. Cells were allowed to migrate for 4 h at 37°C under 5% CO_2. Migrated cells and medium from the lower wells were collected, spun down for 5 min at 1200rpm, 4°C, resuspended in medium and counted using a hemacytometer. The number of cells that migrated in response to unconditioned medium was subtracted from the number of cells migrating in response to conditioned medium to control for nonspecific migration. Recombinant human MIP-3α (Peprotech) and anti-human MIP-3α antibody (R & D Systems) were used in the compensation and neutralization migration assays. Each sample was run in duplicate in each experiment and paired t-test was used to generate p values.

Reporter assay

HFKs were seeded in 6-well dishes at a density of 1×10^5 cells per well. The luciferase reporter construct, kindly provided by Andrew Keates, consisted of an 849-bp fragment of the MIP-3α promoter cloned into the pGL3-Basic firefly luciferase expression vector (MIP-3αWT). A mutant reporter construct was also used which contained 4 nucleotide substitutions in the NF-κB binding element on the promoter (MIP-3αmNFκB). Cells were transfected 24 h later with 100ng MIP-3αWT or MIP-3αmNFκB and 900ng pSG5 using Fugene 6 (Roche). 24 h after transfection, cells were washed with 1x PBS, refed with PSA-free EpiLife, and left untreated or treated with 10μg/mL Poly(I)·Poly(C), 10ng/mL TNF-α, or 10ng/mL IL-1β for 6 h. Following

treatment, cells were harvested, lysed, and measured for luciferase activity as described previously (42, 49). Assays were carried out in triplicate for each sample.

Micro fluidic card analysis

As described previously, 1×10^6 cells were seeded on 10-cm dishes 24 h prior to treatment with Poly(I)·Poly(C). Following treatment and addition of minimal EpiLife, cells were harvested and total RNA was extracted using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNA was provided to the University of Rochester Functional Genomics Center for cDNA amplification and RT-PCR gene expression quantification using micro fluidic cards (Applied Biosystems) containing primers specific for a panel of genes implicated in the immune response. Following sample loading, cards were run on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and data was analyzed using the Sequence Detection System (SDS) 2.2 software using GAPDH as the endogenous control and the control cell line (Babe) set as the calibrator sample (expression equal to 1). Each sample was run in quadruplicate in each experiment and paired t-test was used to generate p values. Results are expressed as the average fold reduction in gene expression for three experiments using three independent cell lines. Paired t test was used to generate p values. Only those targets where fold reduction was statistically significant ($p < 0.05$) are listed in Table 1.

Results

MIP-3α mRNA and protein is decreased in HPV-16 E6/E7-expressing keratinocytes. From microarray analysis performed in order to identify genes differentially regulated in E6/E7-expressing HFKs compared to control cells (50), several genes involved with immune regulation

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(IL-1α, IL-8, MIP-3α, TNF-α) were identified as being down-regulated in the E6/E7-expressing cells. To obtain a more accurate picture of immune response genes that are de-regulated by E6/E7, microfluidic cards of 96 immune-related genes were used for real-time PCR quantitation. HFKs were stably infected with retrovirus carrying either vector alone (Babe) or a vector containing HPV-16 E6 and E7 in tandem (E6/E7). In order to stimulate cytokine/chemokine production, keratinocytes were activated with Poly(I)·Poly(C), which has been shown to efficiently activate keratinocytes (38). The use of Poly(I)·Poly(C), a synthetic double-stranded ribonucleotide, mimics an intermediate of a viral infection and has commonly been used as an inducer of interferons and cytokines *in vitro*. In our hands, the use of Poly(I)·Poly(C) resulted in the strongest stimulation of MIP-3α production, compared to LPS, TNF-α, and IL-1β (data not shown). Control and E6/E7-expressing keratinocytes were activated with Poly(I)·Poly(C) for 16 h, washed, and refed with minimal EpiLife for 4 h. Each cell line of control and E6/E7-expressing keratinocytes was generated by the retroviral infection of pooled neonatal foreskin keratinocytes; thus, each cell line possesses a mixture of unique genetic backgrounds. To control for genetic background effects, three experiments using cells from different pooled foreskins were infected with retrovirus and treated with Poly(I)·Poly(C). Following treatment, cells were harvested and RNA was purified for cDNA amplification. The micro fluidic cards, containing 96 genes involved in the immune response, were run on the ABI PRISM 7900HT Sequence Detection System (SDS) and data was analyzed with SDS 2.2 software using GAPDH as the endogenous control. Results from the micro fluidic cards validated the immune genes known to be down-regulated in E6/E7-expressing keratinocytes from the microarray and also identified additional cytokines, chemokines, receptors, and wound healing factors that were down-regulated in Poly(I)·Poly(C)-stimulated E6/E7-expressing keratinocytes (Table 1).

Many of these genes may not be direct targets of E6/E7. For instance, the expression of RANTES, enthothelin 1 (EDN1), and complement component 3 (C3) in keratinocytes is increased after stimulation with TNF-α (39, 48, 68). IL-1β induces the production of TNF-α, and IL-15 stimulates inducible nitric oxide synthetase (iNos) in keratinocytes (23, 73). It is possible that down-regulation of these genes may be an indirect result of repression of TNF-α, IL-1β, and IL-15 by E6/E7. MIP-3α expression has been shown to up-regulated by TNF-α, IL-1α, and IL-1β (12, 26, 36, 46, 67). Initial experiments using a MIP-3α promoter assay in primary human keratinocytes demonstrated that E6 and E7, both in cooperation and individually, can repress MIP-3α promoter activity even in the presence of exogenously added IL-1β (data not shown). These results suggested that expression of E6/E7 was having a direct effect on MIP-3α transcription. Since MIP-3α was not provided as a target gene on the micro fluidic card, it was necessary to perform separate real-time PCR to validate the repression of MIP-3α observed in the microarray. Additionally, MIP-3α was selected for further investigation due to its selective and potent role in the recruitment of LCs during the innate immune response.

To examine the effect of E6/E7 on MIP-3α mRNA and protein levels, control and E6/E7-expressing cells were stimulated with Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed with PBS, and refed with minimal EpiLife for 4 h. The cells were harvested for evaluation by real-time qPCR, and culture supernatants were collected for use in ELISA analysis and migration assays. Following Poly(I)·Poly(C) treatment of three independent cell lines, E6/E7-expressing keratinocytes show an average 3-fold reduction in MIP-3α mRNA levels compared to control cells (Figure 1A). We next examined the secretion of MIP-3α by Poly(I)·Poly(C)-treated keratinocytes. As shown in Figure 1B, levels of secreted MIP-3α protein

parallel the levels of mRNA expression, such that there was a reduced level of MIP-3α in cells expression E6/E7 (123 pg/mL) compared to controls (228 pg/mL).

Culture supernatant from HPV-16 E6/E7-expressing keratinocytes reduces migration of Langerhans precursor-like cells (LPLCs). To evaluate the effect of factors secreted by the keratinocytes on the migration of LPLCs, the cells needed for the migration assays were first generated from CD14$^+$ monocytes. Dendritic cells can be generated *in vitro* by culturing either CD34$^+$ hematopoietic progenitor cells from bone marrow or cord blood, or peripheral blood CD14$^+$ monocytes with combinations of granulocyte/macrophage colony stimulating factor (GM-CSF), TNF-α, or IL-4 (66). LC precursor cultures, however, require the use of an additional cytokine, transforming growth factor-β1 (TGF-β1), which is critical for the development of LCs *in vitro* and *in vivo* (7, 33). The use of IL-4 suppresses the expression of CCR6 on LCs (13), so peripheral blood CD14$^+$ monocytes cultured in the presence of GM-CSF, IL-4, and TGF-β1, show minimal expression of CCR6 (15) and monocytes cultured in GM-CSF and IL-4 do not express CCR6 (21). In order to generate LPLCs from CD14$^+$ cells, the cells were cultured in GM-CSF and TGF-β1 for 6 days. These culture conditions induced expression of CCR6 and ensured low levels of CD83 and high levels of class II molecules consistent with immature status and ability of the LPLCs to uptake antigen. Results from FACS analysis on CD14$^+$ monocytes cultured in the presence of GM-CSF and TGF-β1 revealed cells that expressed surface markers and antigen uptake characteristics of Langerhans cell precursors: CD14 (47% ± 17), CCR6 (13% ± 8), CD83 (10% ± 4), class II molecules (95% ± 2), antigen uptake (63% ± 12) (Figure 2A) consistent with other studies either using CD14$^+$ monocytes in similar culture conditions or LC-precursors (21, 25). Addition of 100ng/mL LPS to the LPLC

cultures for 24 h resulted in increased expression of CD14 and decreased antigen uptake and expression of class II molecules (data not shown) consistent with the ability of LPLCs to respond to LPS and begin maturation.

After six days in culture, LPLCs were used in Transwell cell culture chambers to determine their migratory response to culture supernatants from Poly(I)·Poly(C)-treated control and E6/E7-expressing keratinocytes. The migratory response of the LPLCs to MIP-3α was first tested to confirm that the level of CCR6 expression generated under the culture conditions described in this study would allow investigation of differences in migration between supernatants from control and E6/E7-expressing keratinocytes. In three independent experiments, the addition of 100ng/mL MIP-3α to minimal media increased migration of LPLCs an average of 2.5-fold (data not shown). The migration of three independent LPLC cultures was then tested against matched supernatant from three independent cell lines of E6/E7-expressing keratinocytes and each migration assay involved duplicates of every test sample. As shown in Figure 2B, exposure to supernatant from E6/E7-expressing keratinocytes resulted in a 1.5–fold reduction in the number of migrating LPLCs (p<0.001).

Decreased migration of LPLCs is specific to the reduction of MIP-3α by HPV-16 E6/E7-expressing keratinocytes. To determine the role of MIP-3α in the migratory response of LPLCs, MIP-3α was added to conditioned media from E6/E7-expressing keratinocytes. The amount of exogenous MIP-3α added to each migration assay was calculated from ELISAs carried out on the culture supernatants from Poly(I)·Poly(C)-treated control and E6/E7-expressing keratinocytes (Figure 1B). The number of migrating LPLCs increased, following addition of exogenous MIP-3α to supernatant from E6/E7-expressing keratinocytes, to the level

of LPLCs migrating in response to supernatant from control cells (Figure 3A). To further

examine the effect of MIP-3α on migrating LPLCs, neutralizing antibody to MIP-3α was added

to supernatant from Poly(I)·Poly(C)-treated control cells. Similar to the compensation assays,

the amount of MIP-3α to be neutralized in the migration assay was calculated from ELISAs

carried out on culture supernatants (Figure 1B). The approximate amount of neutralizing

antibody to be used was calculated using the Neutralization Dose$_{50}$ provided on the technical

data sheet. Use of the neutralizing antibody resulted in a reduction in the migration of LPLCs to

the level of that seen with supernatant from E6/E7-expressing keratinocytes (Figure 3B).

Both E6 and E7 decrease MIP-3α mRNA and protein levels in keratinocytes. In order to

investigate the individual contributions of E6 or E7 to the repression of MIP-3α, cell lines that

expressed each oncoprotein were generated. Retroviral constructs containing stop codons in

either E6 or E7 of HPV-16 were used to produce keratinocytes expressing considerable levels of

E6 alone (E6/E7stop) and E7 alone (E6stop/E7). To determine the effects of E6 or E7 on the

down-regulation of MIP-3α, control, E6/E7, E6, and E7-expressing cells were stimulated with

Poly(I)·Poly(C) as described previously. Following treatment, cells were harvested for

evaluation by real-time qPCR, and culture supernatants were collected for use in ELISA

analysis. The results following treatment of three independent cell lines showed that E6/E7-

expressing keratinocytes had the greatest repression (75%) in MIP-3α mRNA levels compared to

control cells, followed by E6-expressing cells (62%) and E7-expressing cells (58%) (Figure 4A).

Similar to the data shown in Figure 1, the levels of secreted MIP-3α protein parallel the mRNA

levels, with E6/E7-expressing keratinocytes secreting the least amount of MIP-3α (84pg/mL)

compared to control cells (304pg/mL), followed by E6-expressing cells (88pg/mL) and E7-expressing cells (178pg/mL) (Figure 4B).

Decreased MIP-3α expression also occurs in low-risk HPV types. Although not prone to oncogenic transformation of the cells they infect, the capacity for low-risk HPVs to evade the host immune response would be beneficial to the persistence and propagation of the virus. To examine whether suppression of MIP-3α is unique to only the high-risk HPV types, cell lines were generated carrying HPV-16 E6/E7, HPV-6 E6/E7, or HPV-11 E6/E7. The cell lines and control keratinocytes were treated with Poly(I)·Poly(C) as before, then cells and culture supernatants were harvested for qPCR and ELISA analysis. Real-time PCR and ELISA results revealed similar reductions in MIP-3α mRNA levels among all three HPV types, as well as decreased production of MIP-3α protein when compared to control cells (Figures 5A and 5B).

Results showing MIP-3α suppression in cells expressing E6 or E7 individually (Figures 4A and 4B) suggest that both E6 and E7 contribute to the repression of MIP-3α; however, the mechanism underlying this inhibition is not known. For high-risk HPVs, their oncogenic potential has often been linked to the ability of E6 to bind and degrade p53 and of E7 to bind to retinoblastoma (Rb) and abrogate the functions of the Rb family members (27, 30, 51, 59). Therefore, one possible mechanism of the effect of HPV-16 E6 and E7 on MIP-3α expression would be through their interactions with p53 and Rb. However, E6 of HPV-6 and HPV-11 shows no binding to or degradation of p53 (58, 72), and E7 of HPV-6 and HPV-11, while binding to Rb, do so to a lesser extent than that seen with E7 of high-risk HPVs (24, 45). The ability of E6/E7 from high-risk and low-risk HPV types to repress MIP-3α transcription suggests

that the inhibition of MIP-3α is through a mechanism independent of the effects of E6 and E7 on p53 and Rb.

NF-κB is required for MIP-3α expression in keratinocytes. The pro-inflammatory cytokines TNF-α and IL-1β have been shown to induce MIP-3α through NF-κB activation (14, 22, 31, 36, 65). To determine if MIP-3α expression in human keratinocytes requires NF-κB, reporter assays were performed using luciferase constructs containing either the full-length MIP-3α promoter or the full-length MIP-3α promoter containing a substitution mutation in the NF-κB binding site. As shown in Figures 6A and 6B, the mutated NF-κB binding site decreased basal MIP-3α activity as well as induction by Poly(I)·Poly(C), TNF-α, and IL-1β.

The central role of NF-κB in mediating the immune response has been clarified through the investigation of NF-κB activity in response to bacterial and viral infections as well as the regulation of numerous cytokines, chemokines, receptors, wound healing factors, and cell adhesion molecules by NF-κB (17, 47, 57). Activation of transcription by NF-κB is regulated through the recruitment of coactivators such as CBP (CREB-binding protein), p300, and pCAF (p300/CBP-associated factor) (16, 52, 62). HPV-16 E6 has been shown to down-regulate NF-κB activation through its interaction with CBP/p300 (49). This effect of E6 is independent of its ability to degrade p53 (49) and this interaction between E6 and CBP/p300 results in down-regulation of promoters that required NF-κB, such as IL-8, another important chemoattractant of immune cells (28). Additionally, HPV-16 E7 has been shown to bind to pCAF as well as p300 (3, 6, 28) and inhibit its co-activator function.

To test whether the repressive effects of E6 and E7 on MIP-3α are occurring through similar mechanisms, we used a mutant of HPV-16 E6 (E6 C66G/C136G) that was shown

previously not to bind to CBP/p300 (49) and a mutant of HPV-16 E7 (E7.2) that exhibits less

binding to pCAF compared to wild-type HPV-16 E7 (28). In addition to control cells and

E6/E7-expressing keratinocytes, cell lines were generated expressing E6 alone (E6/E7stop), E6

C66G/C136G (E6 C66G/C136G/E7stop), E7 alone (E6stop/E7), and E7.2 (E6stop/E7.2).

Results following Poly(I)·Poly(C) treatment of two independent cells lines showed statistically

significant repression of MIP-3α mRNA in the E6/E7 and E6-expressing keratinocytes compared

to control cells, but MIP-3α mRNA level in the cell line expressing E6 C66G/C136G was close

to the level of control cells (Figure 6C). Similar results were observed when using E6/E7, E7,

and E7.2-expressing keratinocytes (Figure 6D), suggesting that the interaction of HPV-16 E6 and

E7 with CBP/p300 and pCAF contributes to the overall repression of MIP-3α production in

keratinocytes.

Discussion

Although highly correlated with the development of premalignant and malignant disease

of the lower genital tract and oral cavity, infection with high-risk HPVs is not sufficient for the

progression of disease. Many factors, including immune status, contribute to viral persistence

and advancement to more severe stages of disease. The role of the immune system in controlling

HPV infection has been corroborated by studies showing increased prevalence and persistence of

infections in individuals with suppressed immune function, such as transplant recipients and

HIV-positive individuals (63). The squamous epithelium that lines the reproductive tract, the

oral cavity and the skin provides the first line defense against bacterial and viral pathogens. The

initial innate immune response of the skin involves the release of cytokines, chemokines,

antimicrobial molecules, and complement proteins by monocytes, macrophages, professional

antigen presenting cells, and keratinocytes (8, 34, 69). Keratinocytes play an active role in the innate immune response as cellular sources of cytokines in the epithelium (IL-1, -6, -7, -10, -12, -15, -18, -20) which not only influence the migration of immune cells, but also stimulate the further release of cytokines by keratinocytes and immune cells (20). Also important in the initiation of the immune response are chemokines secreted by keratinocytes (IL-8, Gro-α/-β/-γ, IP-10, MCP-1) which are chemotactic for leukocytes, T lymphocytes, monocytes, eosinophils, and basophils (60).

Immune surveillance of the epithelium is maintained by immature Langerhans cells which function as professional antigen-presenting cells and stimulate T lymphocytes following antigen uptake (5, 53). The expression of CCR6 by immature Langerhans cells, or Langerhans precursor-like cells, allows their selective migration in response to MIP-3α (19, 37, 40, 54), a potent chemokine released by inflamed epithelium (9, 46). However, in HPV lesions there is a reduction in many of the immune cells needed to mount an effective immune response. Studies of cervical biopsies revealed that the number of Langerhans cells present in cervical tissue is decreased in low-grade and high-grade cervical intraepithelial neoplasias (CINs) as well as squamous intraepithelial lesions compared to normal tissue (2, 11, 29, 70) which suggests that areas of HPV persistence also have decreased immune surveillance. The reduced levels of immune cells in lesions would allow persistence of the viruses and consequently the lesions for prolonged periods, which appears to be required for the eventual malignant conversion.

Results from this study demonstrate that expression of HPV-16 E6 and E7 in human keratinocytes down-regulates the level of MIP-3α mRNA and protein. The decreased MIP-3α production by E6/E7-expressing keratinocytes reduces the migration of immature Langerhans cell precursors expressing CCR6. Interestingly, the E6/E7 proteins from the low-risk HPV types

6 and 11 are also capable of modulating MIP-3α expression and this may account for the persistence of genital warts in infected individuals. This is a singular instance where the E6 and E7 proteins from low-risk types exhibit a biological behavior that is similar in quality and quantity to that of E6/E7 from high-risk types.

However, E6 and E7 from HPV-16 appear to have an immune-modulatory role that is more broad than the simple effects on MIP-3α, as indicated by the down-regulation of numerous other cytokines, chemokines, wound healing factors, immune receptors, and adhesion molecules presented in this study (Table 1). Many of the immune factors identified in Table 1 bind to receptors found on a variety of immune cells and can, thus, direct the migration and influence the function of these cells. Immature dendritic cells migrate in response to RANTES, IL-8, and MIP-1α (4) during the innate immune response, and T cell responses are regulated by IL-8 and I-CAM (61). The overall reduction in the expression of these and other proteins by E6/E7 may account for the low numbers of various immune cells in lesions caused by the virus, and may further contribute to the ability of the virus to persist unobserved by the immune system. The correlation between HPV expression and a reduction in immune factors is substantiated by studies investigating clinical samples. Studies examining the immune environment of CIN samples compared to normal tissue revealed a decrease in HLA-DR, CD54, TNF-α, GM-CSF, and E-cadherin as well as an increase in IL-10, an immunosuppressive cytokines (18, 23, 29, 44). Additionally, the presence of HPV-16 E6 and E7 has been directly linked to the downregulation of other immune mediators, such as IL-18, IL-8, and MCP-1 (10, 28, 35).

The activation of many immune response genes is mediated by NF-κB, a transcription factor that is a target of bacterial products, viral infections, and pro-inflammatory cytokines such as TNF-α and IL-1β, and acts as a mediator in the expression of immune-related genes. Nearly

two-thirds of the targets identified as being down-regulated in E6/E7-expressing keratinocytes (Table 1) are target genes of the NF-κB signaling cascade (17, 47). HPV-16 E6 and E7 have been shown to bind NF-κB coactivators, p300 and pCAF (3, 6, 28, 49). This interaction of E6 with p300 and E7 with pCAF resulted in decreased IL-8 promoter activity (28) and is implicated in the present study in the repression of MIP-3α transcription. Since E6 and E7 from both high and low-risk viruses modulated MIP-3α production, the mechanisms may be shared between them. The ability of E6 and E7 of low-risk HPV types to also bind p300 and pCAF (3, 6, 28, 45, 49) suggests that the interaction of E6 and E7 with these coactivators could be the mechanism underlying the repression of MIP-3α observed in HPV-16, HPV-6 and HPV-11 E6/E7-expressing cell lines.

In summary, the results reported here concentrated on MIP-3α, a potent chemoattractant for immature LCs, and showed that E6 and E7 from HPV-16 independently reduced transcription. The resulting decrease in MIP-3α production reduced the overall number of LPLCs migrating in response to this immune chemoattractant. Interestingly, the E6/E7 proteins from low-risk types also exhibited the same reduction in MIP-3α. The ability of E6 and E7 to lessen the production of immune mediators, such as MIP-3α, by infected cells would allow the virus to remain undetected and increase the probability of viral persistence and disease progression.

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Figure 1. MIP-3α expression in control and E6/E7-expressing keratinocytes measured by QPCR and ELISA. Primary human foreskin keratinocytes stably expressing vector alone (Babe) or HPV-16 E6/E7 (E6/E7) were treated with 100µg/mL Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. (A) RNA was harvested from the cells and mRNA levels for MIP-3α were determined by qPCR. Results are normalized to GAPDH and expressed as the mean ± SD of three experiments using three independent cell lines (*$p<0.001$). (B) Culture supernatants were harvested and levels of secreted MIP-3α were measured by ELISA. Results are expressed as the mean ± SD of three experiments using three independent cell lines (*$p<0.001$).

Figure 2. Characterization and migration of Langerhans precursor-like cells. (A) Langerhans precursor-like cells (LPLCs) were generated from CD14+ peripheral blood monocytes as described in Materials and Methods. On day 6, LPLCs were stained with anti-CD14, CCR6, CD83, MHCII mAbs or incubated with conjugated ovalbumin and were analyzed with a FACScan using CellQuest software. Open histograms indicate cell staining with the isotype-matched negative control antibody. (B) Primary human foreskin keratinocytes stably expressing vector alone (Babe) or HPV-16 E6/E7 (E6/E7) were treated with Poly(I)·Poly(C). Following treatment, cells were washed with PBS and refed with minimal media for 4 h. Culture supernatants were collected and used in multiwell chamber assays. Values are expressed as migration of LPLCs in response to E6/E7 supernatant relative to migration in response to supernatant from control cells (Babe). Results are expressed as the mean ± SD of three experiments using three independent cell lines (*p<0.001).

Figure 3. Effect of MIP-3α on migration of Langerhans precursor-like cells. Primary human foreskin keratinocytes stably expressing vector alone (Babe) or HPV-16 E6/E7 (E6/E7) were treated with Poly(I)·Poly(C). Following treatment, cells were washed with PBS and refed with minimal EpiLife for 4 h. Culture supernatants were collected and used in multiwell chamber assays. Values are expressed as migration of LPLCs in test samples relative to migration in response to supernatant from control cells (Babe). (A) Migration of LPLCs was increased upon addition of MIP-3α to E6/E7 supernatant. Results are expressed as the mean ± SD of three experiments using three independent cell lines (*p<0.003). (B) Migration of LPLCs was inhibited when MIP-3α neutralizing antibody was added to control (Babe) supernatant. An equivalent concentration of goat IgG was added to duplicate wells as an internal control. Results are expressed as the mean ± SD of three experiments using three independent cell lines (*p<0.05).

Figure 4. Effect of E6 and E7 on MIP-3α mRNA and protein levels. Primary human foreskin keratinocytes stably expressing vector alone (Babe), HPV-16 E6/E7 (E6/E7), HPV-16 E6/E7stop (E6), or HPV-16 E6stop/E7 (E7) were treated with 100μg/mL Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. (A) RNA was harvested from the cells and mRNA levels for MIP-3α were determined by qPCR. (B) Culture supernatants were harvested and levels of secreted MIP-3α were measured by ELISA. Results are expressed as the mean ± SD of three experiments using three independent cell lines (*p<0.001).

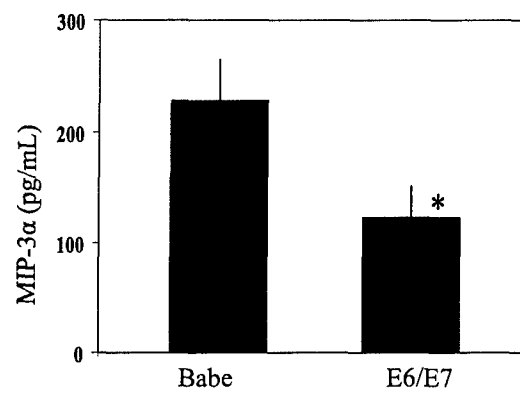**Figure 5. MIP-3α expression in control, HPV-16 E6/E7, HPV-6 E6/E7 and HPV-11 E6/E7-expressing keratinocytes measured by QPCR and ELISA.** Primary human foreskin keratinocytes stably expressing vector alone (Babe), HPV-16 E6/E7 (16-E6/E7), HPV-6 E6/E7 (6-E6/E7), or HPV-11 E6/E7 (11-E6/E7) were treated with 100µg/mL Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed and refed with minimal EpiLife for 4 h. (A) RNA was harvested from the cells and mRNA levels for MIP-3α were determined by qPCR. Results are expressed as the mean ± SD of thre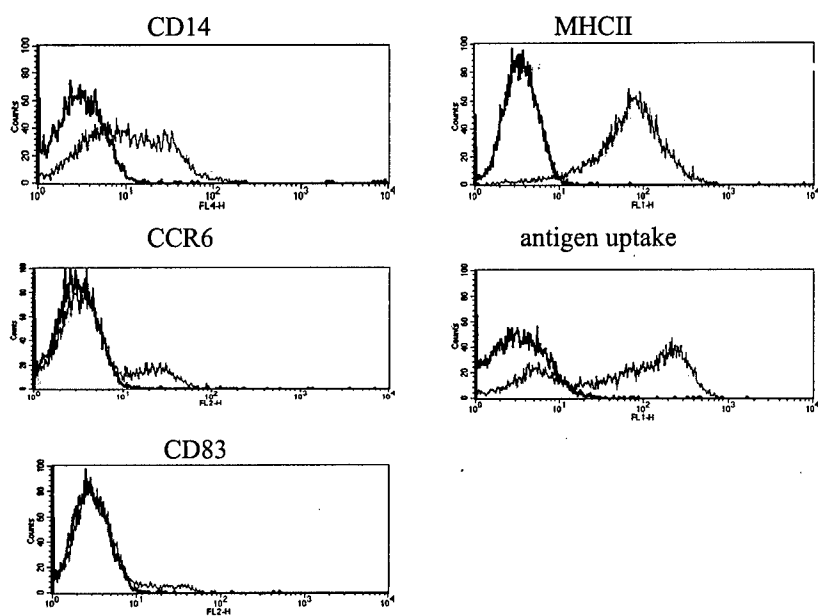e experiments using two independent cell lines (*p<0.001). (B) Cu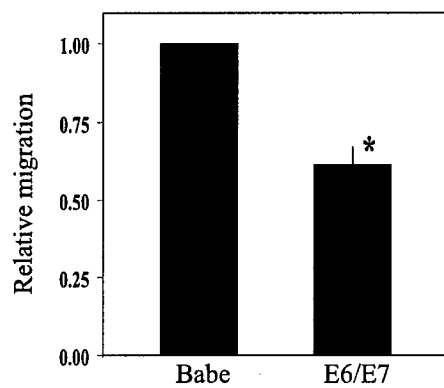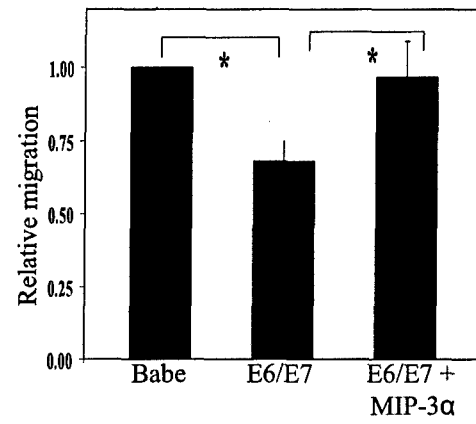lture supernatants were harvested and levels of secreted MIP-3α were measured by ELISA. Results are expressed as the mean ± SD of three experiments using two independent cell lines (*p<0.001).

Figure 6. MIP-3α activity in keratinocytes requires NF-κB signaling. (A, B) 1×10^5

keratinocytes were seeded in 6-well dishes and transfected 24 h later with 100ng MIP-3αWT or

MIP-3αmNFκB using Fugene 6. 24 h after transfection, cells were washed with 1x PBS and

treated with media alone or media containing 10μg/mL Poly(I)·Poly(C), 10ng/mL TNF-α, or

10ng/mL IL-1β for 6 h. Following treatment, cells were harvested, lysed, and measured for

luciferase activity. Results are expressed as the mean RLU ± SD of two independent

experiments. (C,D) Primary human foreskin keratinocytes stably expressing vector alone

(Babe), HPV-16 E6/E7 (E6/E7), HPV-16 E6/E7stop (E6), HPV-16 E6(C66G/C136G)/E7stop

(E6(C66G/C136G)), HPV-16 E6stop/E7 (E7), or HPV-16 E6stop/E7.2 (E7.2) were treated with

100μg/mL Poly(I)·Poly(C) for 16 h. Following treatment, cells were washed and refed with

minimal EpiLife for 4 h. RNA was harvested from the cells and mRNA levels for MIP-3α were

determined by qPCR. Results are expressed as the mean ± SD of three experiments using 3
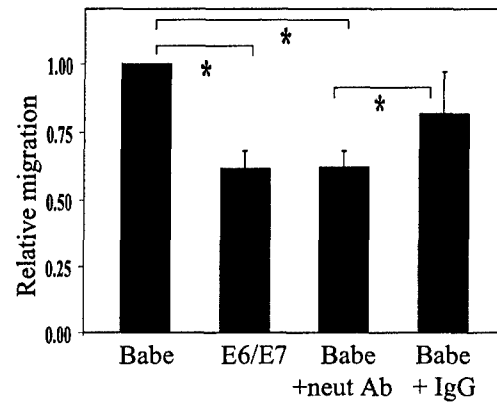
independent cell lines. (*$p < 0.05$).

1A

1B

2A

CD14

MHCII

CCR6

antigen uptake

CD83

2B

3A

3B

4A

4B

5A

5B

6A

Relative luciferase units

Poly(I)·Poly(C)	-	+	-	-
TNF-α	-	-	+	-
IL-1β	-	-	-	+

6B

Relative luciferase units

Poly(I)·Poly(C)	-	+	-	-
TNF-α	-	-	+	-
IL-1β	-	-	-	+

6C

Relative gene expression

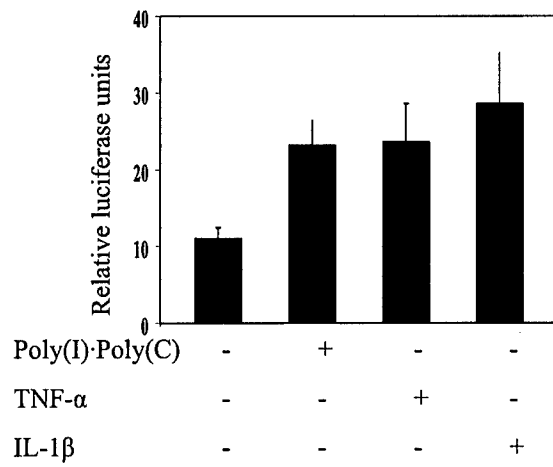Babe   E6/E7   E6   E6 (C66G/C136G)
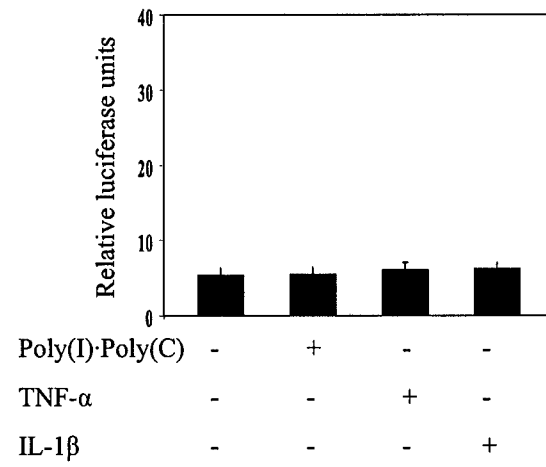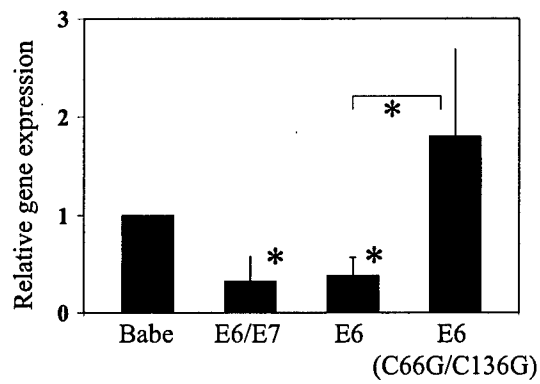
6D

Relative gene expression
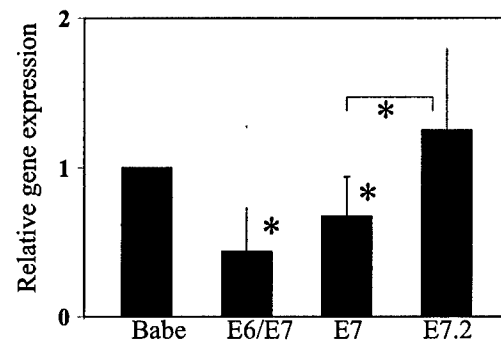
Babe   E6/E7   E7   E7.2
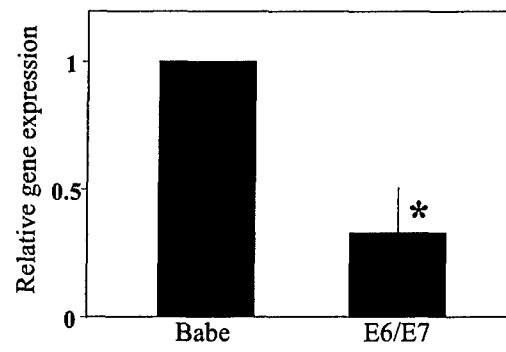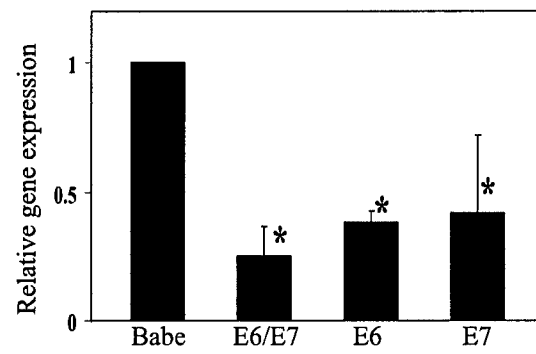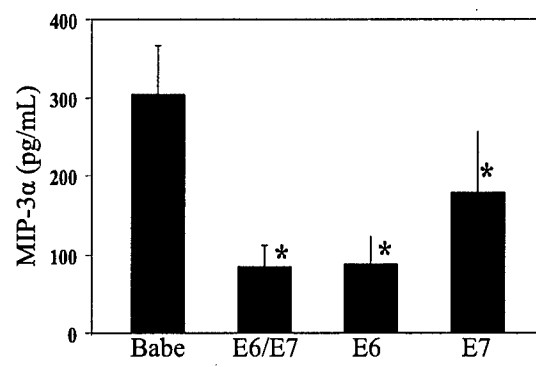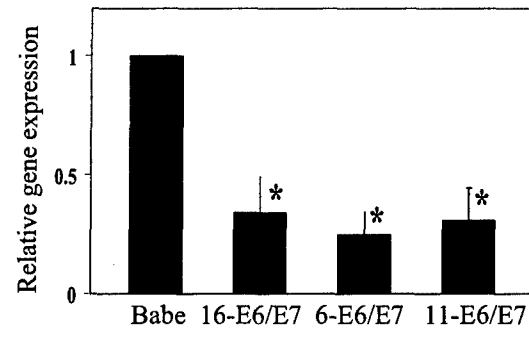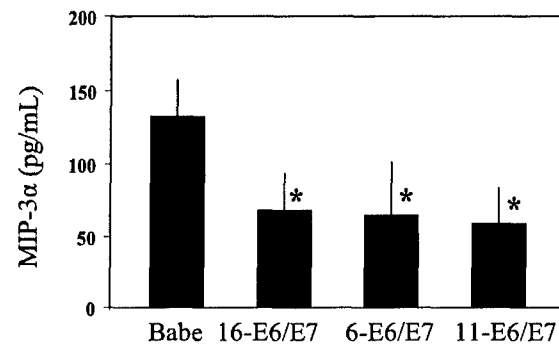
TABLE 1. Immune-related genes down-regulated in E6/E7-expressing keratinocytes.

Gene name	Accession number	Average fold decrease in E6/E7 cells
CD28[a]	NM_006139	50
CD25[a]	NM_000417	25
CD80[a]	NM_005191	20
iNOS	NM_000625	11
CYP7A1	NM_000761	10
LTα	NM_000595	6.7
CD54 (ICAM)	NM_000201	5.9
RANTES	NM_002985	5.6
MIP-1α	NM_002983	4
ITAC	NM_005409	3.6
MIP-3α[b]	NM_004591	3
IP10	NM_001565	3
CD40	NM_001250	3
C3	NM_000064	2.8
IL-8	NM_000584	2.8
FasL	NM_000639	2.6
M-CSF	NM_005211	2.6
IL-15	NM_000585	2.4
HO-1	NM_002133	2.4
ECE-1	NM_001397	2.4
Fas	NM_000043	2.4
EDN1	NM_001955	2.3
IL-6	NM_000600	2.1
NFκB2(p49)	NM_002502	2.1
IL-1β	NM_000576	1.9
MADH-7	NM_005904	1.5
BCL-XL	NM_001191	1.5
Stat3	NM_139276	1.5
BAX	NM_004324	1.4

[a]denotes expression in 2 of 3 micro fluidic cards

[b]not a target gene included on micro fluidic cards; value expressed is a result of separate real-time PCR validation

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